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Silver Conjugated Protein, and  
Antibacterial/Antifungal Agent and Antibacterial/Antifungal  
Paper using the same

5

#### Technical Field

The present invention relates to a novel silver  
conjugated protein having antibacterial/antifungal activity and  
an antibacterial/antifungal agent and an antibacterial/  
10 antifungal paper using the same.

#### Background Art

In recent years, from the viewpoint of health and  
sanitation, a lot of medical apparatuses and instruments,  
15 stationery, textiles, paper products, daily-use goods, bath goods  
and the like are antibacterially/antifungally treated for  
preventing growth of various species of microorganisms such as  
fungi and bacteria. For example, hospitals use plastic  
stationery and instruments which are provided with  
20 antibacterial/antifungal activity for the purpose of preventing  
nosocomial infection. Now higher safety for health is required  
of such articles that directly contact human bodies and food,  
and there is a demand for development of an antibacterial/  
antifungal agent with excellent antibacterial/antifungal activity  
25 and products treated with the agent.

As such antibacterial/antifungal agents, there are proposed antibacterial/antifungal agents wherein antibacterial/antifungal metals such as silver, copper and zinc are adsorbed on water-insoluble scleroproteins (which are proteins contained in eggshell membrane, feathers, wool, silk and the like; and collagen, elastin, silk fibroin and the like separated from such materials) (see Japanese Unexamined Patent Publication Nos. HEI 6(1994)-65013, HEI 8(1996)-188513 and HEI 8(1996)-258235).

These antibacterial/antifungal agents are obtained, for example, by mixing and stirring an eggshell membrane powder with an aqueous solution of silver nitrate, subsequently filtering the precipitate out of the resulting mixture solution, and then washing the precipitate with water, followed by dehydrating and drying (see Japanese Unexamined Patent Publication No. HEI 6(1994)-65013, paragraph [0011], for example).

However, the antibacterial/antifungal agents thus obtained have not been able to provide a sufficient antibacterial/antifungal activity to a variety of articles. It is considered that the reason is that the antibacterial/antifungal agents have low contents of the antibacterial/antifungal metals, and that the binding between the proteins and the antibacterial/antifungal metals is weak physical or ionic binding and therefore the antibacterial/antifungal metals are easily released from the proteins owing to water-washing and the like.

On the other hand, Japanese Pharmacopoeia describes silver protein, a compound of silver and protein. The compound of silver and protein exists as a peptide aggregate composed of water-insoluble Ag conjugated peptides which have  
 5 undergone a secondary structural change owing to their binding to silver ions and water-soluble peptides which do not combine easily to silver ions because the water-soluble peptides do not have intramolecular thiol groups.

Accordingly, if the silver protein is used for other  
 10 applications than medical supplies, the silver protein elutes easily and cannot exhibit a sufficient effect as an antibacterial /antifungal agent.

In addition, a number of inorganic metal compounds containing silver, copper, zinc and the like have been developed  
 15 as active components for antibacterial /antifungal paper. The mechanism of the activity of these inorganic metal compounds is not well known. However, it is considered that the active components absorbed as metal ions in microbial cells inhibit the fundamental metabolism in the respiration and the electron  
 20 transfer sequence of microorganisms or the transfer of substances through cell membrane of microorganisms and thereby exhibit the antibacterial /antifungal activity. However, a problem lies in that active materials composed of the inorganic metal compounds can hardly be applied (fixed) onto paper  
 25 without using a resinous binder.

An object of the present invention is to provide a water-insoluble conjugated protein which contains a high content of silver and from which antibacterial/antifungal silver is not released easily, and an antibacterial/antifungal agent and an antibacterial/antifungal paper using the conjugated protein.

#### Disclosure of Invention

The present invention provides a water-insoluble silver conjugated protein formed of a silver salt and a water-soluble protein containing 0.1 to 200  $\mu$  moles of active thiol groups per gram.

The present invention also provides an antibacterial/antifungal agent containing the above-mentioned silver conjugated protein as an active component.

The present invention also provides an antibacterial/antifungal paper treated with the above-mentioned antibacterial/antifungal agent.

#### Brief Description of Drawings

Fig. 1 is a graph showing a relationship between the concentration of an aqueous solution of silver nitrate in forming a silver conjugated protein in accordance with the present invention and the yield of the obtained silver conjugated protein as well as the content of silver in the obtained silver conjugated

protein (Preparation Example 10);

Fig. 2 is a graph showing a relationship between the concentration of an aqueous solution of silver nitrate in forming a silver conjugated protein in accordance with the present invention and the yield of the obtained silver conjugated protein as well as the content of silver in the obtained silver conjugated protein (Preparation Example 11) ;

Fig. 3 shows the results of a fungal resistance test on a silver conjugated protein ② in accordance with the present invention (Test Example 9);

Fig. 4 shows the results of a fungal resistance test on a silver conjugated protein ③ in accordance with the present invention (Test Example 9);

Fig. 5 shows the results of a fungal resistance test on a known silver-containing antibacterial agent (Zeomic) (Test Example 9);

Fig. 6 shows growth of a fungus where the silver conjugated protein ③ of the present invention is applied on liner paper at a coating weight of  $0.14\text{g/m}^2$  (Test Example 10);

Fig. 7 shows growth of a fungus where the silver conjugated protein ③ of the present invention is applied on liner paper at a coating weight of  $0.42\text{ g/m}^2$  (Test Example 10);

Fig. 8 shows growth of a fungus where the silver conjugated protein ③ of the present invention is applied on liner paper at a coating weight of  $0.14\text{g/m}^2$  (Test Example 12);

Fig. 9 shows growth of a fungus where the silver conjugated protein ③ of the present invention is applied on liner paper at a coating weight of 0.42g/m<sup>2</sup> (Test Example 12);

Fig. 10 shows the surface of copy paper coated with the silver conjugated protein ① of the present invention (Test Example 13);

Fig. 11 shows the surface of copy paper coated with the silver conjugated protein ③ of the present invention (Test Example 13); and

Fig. 12 shows the surface of copy paper coated with the known silver-containing antibacterial agent (Zeomic) (Test Example 13).

#### Best Mode for Carrying Out the Invention

The "active thiol group" of the protein used in the present invention means a mercapto group (- SH) which is capable of reacting easily with an aqueous solution of a silver compound to form a Ag - S bond.

The content of the active thiol groups in the protein of the present invention is 0.1 to 200  $\mu$  mol/g, preferably 5 to 100  $\mu$  mol/g with respect to the weight of the protein.

A content of the active thiol groups smaller than 0.1  $\mu$  mol/g is not preferable because only a small amount of silver binding to the active thiol groups, and therefore, a desirable water-insoluble silver conjugated protein cannot be obtained.

Also, a content of the active thiol groups larger than 200  $\mu$  mol/g is not preferable because a water-insoluble conjugated compound having a localized binding of silver to the active thiol groups may precipitates. That is, this phenomenon results in a decrease in the amount of silver binding.

The content of the active thiol groups can be determined as L-cysteine equivalent by a DTNB method (Ellman's method) by preparing an aqueous solution of protein quantified beforehand (see Biochemical Experiment 10 "Quantitative Determination of SH group," published by the Academic Publication Center, 1981, p.p. 86 to 93).

The "water-soluble protein having an active thiol group content of 0.1 to 200  $\mu$  mol/g" used in the present invention is not particularly limited so long as the content of the active thiol groups is within the above-mentioned range. Examples thereof include whey protein, a hydrolysate of whey protein, a water-solubilized product of whey protein, a hydrolysate of eggshell membrane protein and a water-solubilized preparation of eggshell membrane protein. These can be suitably used.

The above-mentioned water-soluble proteins retain an emulsification property which is a characteristic of starting proteins. Antibacterial/antifungal agents prepared from these water-soluble proteins are expected to have good adherence to an object.

It should be understood that the "water-solubility" of

the water-soluble proteins in the present invention has a meaning corresponding to "solubility" of soluble proteins in the field of protein.

5 The "water-insolubility" of the water-insoluble silver conjugated protein in the present invention means that the protein is hardly dissolved in water or is not dissolved in water essentially, in contrast to the above-mentioned "solubility."

10 The "whey protein" is contained in a large amount in whey produced as a by-product during production of cheese and essentially contains cystine and the active thiol groups in a relatively large amount. It is possible to obtain a protein containing more active thiol groups by alkali-lysis, enzymatic lysis or treatment with a reducing agent of the whey protein.

15 The whey protein, which contains water-soluble proteins such as  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin, exists richly in whey produced to be a by-product during the production of cheese and can be industrially purchased.

20 A commercially available whey protein, for example, is Sunlacto N-5 (tradename) produced by Taiyo Kagaku Co., Ltd., Japan. The content of the active thiol groups therein is about  $50 \mu \text{mol/g}$ .

25 The whey protein commonly contains reduction sugar like lactose and minerals which are derived from a raw material. Since silver ions may be separated as a metal owing to the presence of lactose, lactose is preferably removed before the



whey protein is contacted to silver ions. Lactose may be removed by dissolving the whey protein in deionized water and dialyzing the resulting solution against deionized water.

As a protein containing a lot of intramolecular cystine,  
5 may be mentioned scleroprotein keratin forming hair, wool and feathers. However, it is difficult to treat hair and feathers as industrial materials as in the present invention. Wool has a large cystine content, but is disadvantageous for the present invention from the viewpoint of costs.

10 Eggshell membrane protein is a material having a cystine content comparable to that of wool protein, and a hydrolysate or a water-solubilized product thereof is suitably used as a protein of the present invention.

The "eggshell membrane protein" is a water-insoluble  
15 protein forming inner membrane of eggshells of birds. The eggshell membrane used in the present invention is preferably made from materials such as chicken eggs or quail eggs which are mass-consumed in the food industry and the like.

This water-insoluble eggshell membrane protein can be  
20 made into a water-soluble hydrolysate or a water-solubilized product containing a specific amount of the active thiol groups by subjecting the protein to alkali-lytic, enzymatically lytic or reducing treatment. That is, the treatment is for cleaving a disulfide bond of the eggshell membrane protein to form the  
25 active thiol group. It is possible to adjust the thiol group

content in the protein by selecting treatment conditions.

Now the collection of eggshell membrane from eggs and the hydrolysis and water-solubilization of the eggshell membrane are described more particularly.

## 5 (Separation of Eggshell Membrane)

An egg consists of an eggshell, eggshell membrane, albumen and yolk sequentially from the outside. The eggshell in the outermost layer closely contacts the eggshell membrane. In order to collect the eggshell membrane, typically, the eggshell  
10 and eggshell membrane are first separated from the albumen and the yolk by breaking an egg. Then the eggshell membrane is peeled off the eggshell, for example, with use of tweezers.

In order to collect a large amount of eggshell membrane industrially, the eggshell and eggshell membrane closely  
15 contacting each other are treated with an acid (e.g., a hydrochloric acid of about 10 % concentration) to dissolve calcium carbonate which is a major component of the eggshell. Calcium carbonate is then discarded by filtration.

For effective treatment with the acid, the eggshell and  
20 eggshell membrane closely contacting each other are preferably ground mechanically beforehand. Further, it is more preferably to grade the resulting ground product, classify it according to difference in specific gravity and treat it with the acid (see Japanese Unexamined Patent Publication No. HEI  
25 3(1991)-45264).

The eggshell membrane separated and collected by the method described in this publication is applied as a seasoning called "Ransho (egg source)" in which the eggshell membrane is decomposed into amino acid, dipeptide or tripeptide and as an ingredient in a cosmetic for activating epidermal cells or fibroblasts as a water-soluble protein.

Next, the eggshell membrane obtained by the above-described method is subjected to the alkali-lysis, enzymatic lysis or reducing treatment as mentioned above to form a water-soluble hydrolysate or a water-solubilized material containing a desired content of the activated thiol groups. Among these treatments, the alkali-lysis is industrially preferable.

#### **(Alkali-lysis)**

The eggshell membrane is treated in an about 1 to 30 % aqueous solution (water or an aqueous solution of ethanol concentration of 40 %) of a hydroxide of an alkali metal (e.g., sodium hydroxide or potassium hydroxide). The concentration of the hydroxide of the alkali metal is selected as appropriate depending upon the amount of the eggshell membrane, the treatment temperature and the like. For example, if the amount of the eggshell membrane is about 50 g, the eggshell membrane is treated with 1000 mL of an aqueous solution of the hydroxide of the alkali metal adjusted to 1 N.

The eggshell membrane to which the aqueous solution of the hydroxide of the alkali metal is added is mixed and stirred

for promoting the alkali-lysis. The treatment temperature is about 40 to 80 °C, and the treatment time is about 3 to 24 hours.

The aqueous solution after the treatment is filtered and the resulting filtrate is dialyzed against deionized water, to give  
5 an hydrolysate of the eggshell membrane protein.

### **(Enzymatic lysis)**

An hydrolysate is obtained by treating the eggshell membrane with a proteolytic enzyme.

As proteolytic enzymes, may be mentioned plant-origin  
10 proteolytic enzymes such as papain and bromelain and animal-origin proteolytic enzymes such as pancreatin, rennin, trypsin, chymotrypsin and pepsin, which are suitably used.

This treatment is carried out in a liquid in which the eggshell membrane is dispersed in water. The treatment  
15 temperature and pH are not particularly limited and may be the optimal temperature and pH of the enzyme used. For example, if pancreatin is used, the temperature is suitably 35 to 50 °C, and the pH is suitably about 6 to 8.

The solution after the treatment is filtered and the  
20 resulting filtrate is dialyzed against deionized water, to give an hydrolysate of the eggshell membrane protein.

### **(Treatment with Reducing Agent)**

A water-solubilized material can be prepared by treating the eggshell membrane with an reducing agent. By  
25 this method, the disulfide bond in the eggshell membrane is

reduced with a reducing agent such as sodium sulfide,  
thioglycolic acid,  $\beta$ -thiopropionic acid or an alkali salt thereof,  
2-mercaptoethanol or the like. The amount of the reducing  
agent used depends on the kind of the agent, but for example, if  
5  $\beta$ -thiopropionic acid is used, the amount of an aqueous  $\beta$ -  
thiopropionic acid solution adjusted to 5N is about 2,000 mL  
with respect to 100g of the eggshell membrane.

This treatment is carried out in water phase suspending  
the eggshell membrane. If  $\beta$ -thiopropionic acid is used as a  
10 reducing agent, the treatment temperature is suitably 60 to 80°C  
and the treatment time is suitably about 5 hours.

The solution after the treatment is filtered and the  
resulting filtrate is dialyzed against deionized water, to give an  
hydrolysate of the eggshell membrane protein.

15 The aforesaid whey protein as it is may be used as a  
water-soluble protein, but may be used as a hydrolysate or a  
water-solubilized material thereof obtained by the above-  
described alkali-lysis, enzymatic lysis or treatment with the  
reducing agent.

20 The "water-insoluble silver conjugated protein" of the  
present invention may be obtained by contacting a water-soluble  
protein having an active thiol group content of 0.1 to 200  $\mu$   
mol/g with a silver salt in water.

The silver salt is not particularly limited so long as it  
25 releases silver ions in water to allow easy binding of silver to

the protein. More particularly, may be mentioned inorganic acid salts such as silver nitrate, silver nitrite, silver sulfate and silver chloride, organic acid salts such as silver acetate, silver lactate and silver oxalate, complex salts such as diamine  
 5 silver nitrate and diamine silver sulfate, and the like. Silver nitrate and silver acetate are particularly preferable from the viewpoint of solubility to water.

The protein may be contacted to the silver salt in water by use of a known process of mixing/stirring, oscillation or the  
 10 like, among which mixing/stirring is industrially preferable.

Particular processes using stirring are:

- ① a process of mixing and stirring the protein and the silver salt in water at once;
- ② a process of adding a silver salt dissolved in water  
 15 little by little into the protein in water under stirring to raise the silver ion concentration in water;
- ③ a process of adding a finely ground silver salt little by little into the protein in water under stirring to raise the silver ion concentration in water;
- 20 ④ a process of adding an aqueous solution of the protein little by little into an aqueous silver salt solution under stirring, finally to keep constant the silver ion concentration in water; and the like.

Among these processes, process ② is particularly  
 25 preferred because the silver conjugated protein can be obtained

with good reproducibility in a high yield.

The proportion of the silver salt to the protein is preferably about 0.2 to 3 g of the silver salt with respect to 1 g of the protein, although it depends upon the contact conditions.

5 More particularly, it is preferable to use about 1,000 mL of an aqueous silver salt solution having a concentration of about 5 to 250 mM with respect to 1,000 mL of an aqueous protein solution having a concentration of 1 to 20 mg/mL. However, the liquid amount ratio of the aqueous silver salt solution to the aqueous  
10 protein solution is not particularly limited so long as a conjugated protein having a high silver content is effectively obtained.

Further, the conditions for contacting the protein to the silver salt may be such that the protein and the silver are  
15 uniformly mixed and a conjugated protein having a high silver content is obtained effectively. For example, in the case of contact by stirring, the treatment temperature is suitably about 0 to 70°C, and the treatment time is suitably within 24 hours.

The mixture solution after reaction is filtered and the  
20 residue is washed with deionized water, ethanol and the like and dried to give a silver conjugated protein.

The silver content in the resulting conjugated protein can be determined, for example, by quantifying silver eluted using a 3 % nitric acid.

25 According to the present invention, there are provided

an antibacterial/antifungal agent containing the above-described silver conjugated protein as an active component and an antibacterial/antifungal paper treated with the antibacterial/antifungal agent.

5 Medical apparatuses and instruments, stationery, textiles, paper products, daily-use goods, bath goods and the like can exhibit the antibacterial/antifungal activity by being provided with the antibacterial/antifungal agent of the present invention by know processes. Among these object articles, the  
10 paper products, in particular, can exhibit excellent antibacterial/antifungal activity.

In the present invention, "paper" means not only sheet-form materials obtained by dispersing cellulose fibers from plants in water, followed by wet papermaking, but also  
15 sheet-form materials such as synthetic paper having fiber-entangling structure and properties similar to paper. More particularly, may be mentioned recycled paper from newspaper and board paper, printing paper, copy paper and liner paper.

As processes for treating paper with the antibacterial  
20 /antifungal agent of the present invention, may be mentioned a process of coating the surface of paper with the silver conjugated protein dispersed in a suitable amount of water or an aqueous medium, a process of impregnating paper with the silver conjugated protein dispersed in a suitable amount of water or an  
25 aqueous medium by immersion or spray (shower) and a process



of making paper from pulp slurry mixed with the silver conjugated protein. The process of coating is particularly preferred because an existing apparatus for papermaking can be converted.

5 In the case where paper is coated with the antibacterial /antifungal agent of the present invention, a preferable coating weight varies depending upon the kind of paper, its use and/or the like, but typically, is 0.01 to 10 g, more preferably 0.1 to 5 g, per square meter. If the coating weight is smaller than 0.01 g  
10 per square meter, it is not preferable because a sufficient antibacterial/antifungal effect cannot be obtained. If the coating weight is larger than 10 g per square meter, it is not preferable because a further antibacterial/antifungal effect cannot be expected and the costs rise.

15

## Examples

The present invention is now described with preparation examples and test examples, but these preparation and test examples should not be construed to limit the present  
20 invention.

### Preparation Example 1 (Preparation of hydrolysate of eggshell membrane protein by alkali-lysis)

A 500 mL round flask was charged with a water-  
25 containing weight of 74.36 g (a dry weight of 10.81 g) of eggshell

membrane, 130 mL of a 2N NaOH aqueous solution and 86 mL of ethanol, which were stirred at 70°C for 64 hours. Then the mixture solution was filtered and the resulting filtrate was dialyzed against deionized water to give 188 mL of an aqueous solution of a hydrolysate of eggshell membrane protein (referred to as "solubilized protein A" hereinafter).

The protein concentration in the solubilized protein A was determined by the Lowry method, and the active thiol group content in the solubilized protein A was calculated from the protein concentration according to the aforesaid "Quantitative Determination of SH group."

The obtained results are shown below. A protein concentration determined by the Burette method and absorbance (280 nm) are also shown for reference. Numerals in parentheses represent converted total-amount values (total absorbance for the absorbance).

	Liquid amount	188 mL
	Active thiol group content	17 $\mu$ mol/l (3.2 $\mu$ mol)
	Protein concentration	
20	by Lowry Method	3.4 mg/mL (639.2 mg)
	by Burette Method	3.8 mg/mL (714.4 mg)
	absorbance	6.2 (1165.6)

The active thiol group content in the solubilized protein A obtained from the above-mentioned values is 4.5 to 5.0 mol/g.

Preparation Example 2 (Preparation of hydrolysate of eggshell membrane protein by alkali-lysis)

A 500 mL round flask was charged with a water-containing weight of 79 g (a dry weight of 11.46 g) of eggshell membrane, 137 mL of a 2N NaOH aqueous solution and 92 mL of ethanol, which were stirred at 70°C for 96 hours. Then the mixture solution was filtered and the resulting filtrate was dialyzed against deionized water to give 640 mL of an aqueous solution of a hydrolysate of eggshell membrane protein (referred to as "solubilized protein B" hereinafter).

As in Preparation Example 1, the protein concentration in the solubilized protein B was determined and the active thiol group content in the solubilized protein B was calculated from the protein concentration. The obtained results are shown below.

	Liquid amount	640 mL
	Active thiol group content	55 $\mu$ mol/l (35.2 $\mu$ mol)
	Protein concentration	
	by Lowry Method	8.0 mg/mL (5120 mg)
20	by Burette Method	7.2 mg/mL (4608 mg)
	absorbance	15.99 (10231)

The active thiol group content in the solubilized protein B obtained from the above-mentioned values is 6.9 to 7.6 mol/g.

25 Preparation Example 3 (Preparation of silver conjugated protein

[illegible]

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acid. The silver content was 4.75 wt%.

15

using solubilized protein B)

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in the same manner as in Preparation Example 3 except that the solubilized protein B was used instead of the solubilized protein A.

The silver content in the silver conjugated protein ②  
5 was 7.0 wt%.

Also the silver ion concentration in the filtrate was determined, and the silver content in the silver conjugated protein ② was calculated from the obtained concentration to be 10.8 wt%. This shows that few silver was released from the  
10 silver conjugated protein ② by washing after the filtration, as in Preparation Example 3.

Preparation Example 5 (Preparation of silver conjugated protein using whey protein)

15 In a 300 mL beaker, 2 g of whey protein (tradename: Sunlacto N-5 produced by Taiyo Kagaku Co., Ltd., with a protein content of 72 % and an active thiol group content of 47  $\mu$  mol/g) were dissolved in 200 mL of deionized water. To this mixture solution, 200 mL of a 50 mM aqueous solution of silver nitrate  
20 were added and stirred for an hour. The resulting mixture solution was allowed to stand overnight and filtered (using a filter paper No.2).

The residue separated by filtration was washed twice with 100 mL of deionized water and dried to give 1646 mg of a  
25 silver conjugated protein ③ (a yield of 115 % with respect to the

protein used).

The silver content in the silver conjugated protein ③ was determined by quantifying eluted silver using a 3 % nitric acid. The silver content was 4.25 wt%.

5 Also the silver ion concentration in the filtrate was determined, and the silver content in the silver conjugated protein ③ was calculated from the obtained concentration to be 4.86 wt%. This shows that few silver was released from the silver conjugated protein ③ by washing after the filtration, as  
10 in Preparation Example 3.

Preparation Example 6 (Preparation of silver conjugated protein using whey protein)

In a 300 mL beaker, 2 g of whey protein (tradename: Sunlacto N-5 produced by Taiyo Kagaku Co., Ltd., with a protein  
15 content of 72 % and an active thiol group content of  $47 \mu\text{mol/g}$ ) were dissolved in 180 mL of deionized water. This mixture liquid was dialyzed against deionized water to remove lactose.

20 mL of a 10 mM aqueous solution of silver nitrate were added to 20 mL of the collected dialysate and the mixture  
20 was stirred for an hour. The resulting mixture solution was allowed to stand overnight and filtered (using a filter paper No.2).

The residue separated by filtration was washed twice  
25 with 50 mL of deionized water and dried to give 154.8 mg of a

silver conjugated protein ④.

The silver content in the silver conjugated protein ④ was determined by quantifying eluted silver using a 3 % nitric acid. The silver content was 7.80 wt%.

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Preparation Example 7 (Preparation of silver conjugated protein using whey protein)

In a 5 L beaker, 30 g of whey protein (tradename: ALACEN 895 produced by New Zealand Milk Product, with a  
10 protein content of 86.5 % and an active thiol group content of 34.5  $\mu$  mol/g) were dissolved in 3 L of deionized water.

To the aqueous solution of the whey protein, was added an aqueous solution of 4.574 g of silver nitrate which had been dissolved in about 300 mL of deionized water in a separate  
15 beaker. The mixture was stirred for an hour. The resulting mixture solution was allowed to stand overnight and spray-dried.

The spray drying was performed with an L-8 type spray drier (produced by Okawahara Kakoki Kabushiki Kaisha) at a  
20 flow rate of 2 L/ hour under the conditions of a hot-air inlet temperature of 180°C and an air outlet temperature of 95°C, to give a silver conjugated protein ⑤.

After the obtained silver conjugated protein ⑤ was decomposed, the silver content was determined by the  
25 ammonium thiocyanate titration method described in Japanese

Pharmacopoeia. The silver content was 9.30 wt%.

Preparation Example 8 (Preparation of silver conjugated protein using whey protein)

5 In a 5 L beaker, 25 g of whey protein (tradename: ALACEN 895 produced by New Zealand Milk Product, with a protein content of 86.5 % and an active thiol group content of 34.5  $\mu$  mol/g) were dissolved in 2.5 L of deionized water.

To the aqueous solution of the whey protein, was added  
10 an aqueous solution of 21.24 g of silver nitrate which had been dissolved in about 2.5 L of deionized water in a separate beaker. The mixture was stirred for an hour. The resulting mixture solution was allowed to stand overnight and spray-dried in the same manner as in Preparation Example 3, to give a silver  
15 conjugated protein ⑥.

After the obtained silver conjugated protein ⑥ was decomposed, the silver content was determined by the ammonium thiocyanate titration method described in Japanese Pharmacopoeia. The silver content was 31.15 wt%.

20

Preparation Example 9 (Preparation of silver conjugated protein using eggshell membrane)

About 40 g of wet weight of raw eggshell membrane (having an active thiol group content of 5  $\mu$  mol/g) separated  
25 from raw eggs are immersed in 800 mL of a 50 mM aqueous



solution of silver nitrate, dispersed by a mixer and allowed to stand overnight. Thereafter, eggshell membrane was collected by filtration and washed with 800 mL of deionized water. The filtering and washing were repeated three times. The eggshell  
 5 membrane after washing was dispersed in 400 mL of deionized water, and wet-ground by a rotary ball mill. Grinding was continuously performed until the average particle diameter in the slurry became smaller than 20 to 30 microns. Thereafter, the slurry liquid was collected, and the ground product was  
 10 filtered, washed and dried by heat under reduced pressure, to give a silver conjugated protein ⑦.

After the obtained silver conjugated protein ⑦ was decomposed, the silver content was determined by the ammonium thiocyanate titration method described in Japanese  
 15 Pharmacopoeia. The silver content was 6.36 wt%.

Comparative Preparation Example 1 (Preparation of silver conjugated protein using mechanically ground eggshell membrane)

20 In a 50 mL beaker, 2 g of a mechanically ground eggshell membrane ① (tradename : Sunkakumaku P produced by Taiyo Kagaku Co., Ltd., the active thiol group content cannot be determined) was suspended by adding 10 ml of a 0.2 % aqueous solution of silver nitrate. This suspension was stirred  
 25 at room temperature for 10 minutes and filtered (using a filter

paper No.2).

The residue separated by filtration was washed three times with 50 mL of deionized water and dried to give 1904 mg of a silver-adsorbing eggshell membrane powder ①.

5 The silver content in the silver-adsorbing eggshell membrane powder ① was determined by quantifying eluted silver using a 3 % nitric acid. The silver content was 0.059 wt%.

Also the silver ion concentration in the filtrate was  
10 determined, and the silver content in the silver-adsorbing eggshell membrane powder ① was calculated from the obtained concentration to be 0.68 wt%. This shows that silver was released from the silver-adsorbing eggshell membrane powder ① by washing after the filtration.

15 Comparative Preparation Example 2 (Preparation of silver conjugated protein using mechanically ground eggshell membrane)

1928 mg of a silver-adsorbing eggshell membrane  
20 powder ② (the active thiol group content was not able to be determined) was obtained in the same manner as in Comparative Preparation Example 1 except that a mechanically ground eggshell membrane ② obtained by grinding with a stone mill and by air-grinding/gradation was used instead of the  
25 mechanically ground eggshell membrane ①.

The silver content in the silver-adsorbing eggshell membrane powder ② was 0.026 wt%.

Also the silver ion concentration in the filtrate was determined, and the silver content in the silver-adsorbing eggshell membrane powder ② was calculated from the obtained concentration to be 0.67 wt%. This shows that silver was released from the silver-adsorbing eggshell membrane powder ② by washing after the filtration.

10 Test Example 1 (Test of silver conjugated proteins on their antibacterial power against Staphylococcus aureus)

The silver conjugated protein ① and the silver conjugated protein ③ were tested on their antibacterial power against Staphylococcus aureus under the following conditions.

15 Known silver-containing antibacterial agents were also tested.

The known silver-containing antibacterial agents were as follows:

Zeomic [tradename (registered trademark), produced by SINANEN ZEOMIC CO., LTD., Japan, containing about 2.5 wt% silver]

Novaron [tradename (registered trademark, produced by Toagosei Co., Ltd., Japan]

The obtained results are shown in Table 1. The numerical values in the table represent viable cell counts, each of which is the average of two samples. Accordingly, two viable

cell count data are shown in a "not added" row. In the following tests, likewise, the viable cell count represents the average of viable cell counts in two samples.

Determination method : in accordance with the  
 5 determination of the minimum bactericidal concentration (MBC) of Antibacterial Evaluation Tests For Inorganic Antibacterial Agents Such As Silver II (1995 edition, edited by the Society for the Study of Inorganic Antibacterial Agents Such As Silver)

Test bacteria : Staphylococcus aureus IFO 12732

10 Media used : SCD medium

SCD agar medium.

The minimum bactericidal concentrations (MBC values) of the silver conjugated protein ① and the silver conjugated protein ③ against Staphylococcus aureus were 3.13 ppm and  
 15 6.25 ppm, respectively.

Table 1

Concentration of agent added (ppm)	Silver conjugated protein ①	Silver conjugated protein ③	Zeomic	Novaron	Concentration of Ag in silver nitrate (ppm)	Silver nitrate
Not added	$8.19 \times 10^5$ $6.90 \times 10^5$	ditto	ditto	ditto	ditto	ditto
3200	0	0	0	$\infty$	6.25	0
1600	0	0	0	$\infty$	3.13	0
800	0	0	0	$\infty$	1.56	0
400	0	0	0	$\infty$	0.78	1
200	0	0	0	$\infty$	0.39	$\infty$
100	0	0	0	$\infty$	0.2	$\infty$
50	0	0	0	$\infty$	0.1	$\infty$
25	0	0	0	$\infty$	0.05	$\infty$
12.5	0	0	296	$\infty$	0.025	$\infty$
6.25	0	132	32	$\infty$	0.013	$\infty$

\* Test bacteria : Staphylococcus aureus

\* It is judged that bacteria has not grown where the viable cell count is not more than 5.

- 5 \* The final concentration of an agent is half the concentration of the agent added because the agent is mixed with the bacteria in equal amounts.

Test Example 2 (Test of silver conjugated proteins on their antibacterial power against Escherichia coli)

The silver conjugated protein ①, the silver conjugated protein ② and the silver conjugated protein ③ were tested on their antibacterial power against *Escherichia coli* under the following conditions. As in Test Example 1, comparative test  
 5 was also carried out with regard to the known silver-containing antibacterial agents. The agents were added in concentrations within the range of 3.13 to 800.00 ppm.

The obtained results are shown in Table 2. The numerical values in the table represent viable cell counts.

10 Determination method : in accordance with the determination of the minimum bactericidal concentration (MBC) of Antibacterial Evaluation Tests For Inorganic Antibacterial Agents Such As Silver II (1995 edition, edited by the Society for the Study of Inorganic Antibacterial Agents Such As Silver)

15 Test bacterial : *Escherichia coli* IFO 3972

Media used : Normal bouillon medium (NB medium)

Normal bouillon agar medium (NA medium).

The minimum bactericidal concentrations (MBC values)  
 20 of the silver conjugated protein ①, the silver conjugated protein ② and the silver conjugated protein ③ against *Escherichia coli* were less than 1.65 ppm, 1.65 ppm and 6.25 ppm, respectively.

That of Zeomic was 6.25 ppm, and that of Novaron was 50 ppm.

[illegible]5

\* It is judged that bacteria has not grown where the viable cell count is not more than 5.

The silver conjugated protein ⑤, the silver conjugated protein ⑥ and the silver conjugated protein ⑦ were tested on their antibacterial power against Escherichia coli under the same conditions as in Test Example 2. The agents were added in concentrations within the range of 0.049 to 25.000 ppm.

10





The mechanically ground eggshell membrane ①, the silver-adsorbing eggshell membrane powder ①, the mechanically ground eggshell membrane ② and the silver-adsorbing eggshell membrane powder ② were tested on their antibacterial power against Staphylococcus aureus under the following conditions.

Determination method : in accordance with the determination of the minimum bactericidal concentration (MBC) of Antibacterial Evaluation Tests For Inorganic Antibacterial Agents Such As Silver II (1995 edition, edited by the Society for the Study of Inorganic Antibacterial Agents Such As Silver)

Test bacterial : Staphylococcus aureus IFO 12732

Media used : SCD medium

SCD agar medium.

The antibacterial power test was carried out with varying the concentrations of the agents within the range of 1.56 ppm to 400 ppm. The antibacterial power was not recognized with any of the mechanically ground eggshell membrane ①, the silver-adsorbing eggshell membrane powder ①, the mechanically ground eggshell membrane ② and the silver-adsorbing eggshell membrane powder ②.

Preparation Example 10 (Effect of the concentration of silver nitrate on the formation of silver conjugated protein)

Silver conjugated proteins were prepared in the same





[illegible]

Concen- tration of agent added (ppm)	Silver conjugated protein ②	Zeomic	Novaron	Concen- tration of Ag in silver nitrate (ppm)	Silver nitrate
Not added	$2.65 \times 10^6$ $3.76 \times 10^6$	ditto	ditto	ditto	ditto
3200	0	0	$\infty$	6.25	0
1600	0	0	$\infty$	3.13	0
800	0	0	$\infty$	1.56	0
400	0	3	$\infty$	0.78	1
200	0	1	$\infty$	0.39	$\infty$
100	0	5	$\infty$	0.2	$\infty$
50	0	16	$\infty$	0.1	$\infty$
25	0	$\infty$	$\infty$	0.05	$\infty$
12.5	0	$\infty$	$\infty$	0.025	$\infty$
6.25	0	$\infty$	$\infty$	0.013	$\infty$

\* Test bacteria : Staphylococcus aureus

\* It is judged that bacteria has not grown where the viable cell count is not more than 5.

5    \* The final concentration of an agent is half the concentration  
of the agent added because the agent is mixed with the bacteria  
in equal amounts.

Test Example 6 (Comparative test of antibacterial power of silver  
10 conjugated protein and known silver antibacterial agents)

### Comparative test of the antibacterial power of the silver

conjugated protein ③ (having a silver content of 4.25 wt%) and known silver-containing antibacterial agents against *Staphylococcus aureus* was carried out under the same conditions as in Test Example 1.

5           The known silver-containing antibacterial agents were aforesaid Zeomic and Novaron, and silver nitrate was also tested for reference.

The obtained results are shown in Table 5. The numerical values in the table represent viable cell counts.

10

Table 5

Concentration of agent added (ppm)	Silver conjugated protein ③	Zeomic	Novaron	Concentration of Ag in silver nitrate (ppm)	Silver nitrate
Not added	$2.65 \times 10^6$ $3.76 \times 10^6$	ditto	Ditto	ditto	ditto
3200	0	0	$\infty$	6.25	0
1600	0	0	$\infty$	3.13	0
800	0	0	$\infty$	1.56	0
400	0	3	$\infty$	0.78	1
200	0	1	$\infty$	0.39	$\infty$
100	0	5	$\infty$	0.2	$\infty$
50	0	16	$\infty$	0.1	$\infty$
25	1	$\infty$	$\infty$	0.05	$\infty$
12.5	20	$\infty$	$\infty$	0.025	$\infty$
6.25	$\infty$	$\infty$	$\infty$	0.013	$\infty$

\* Test bacteria : *Staphylococcus aureus*

\* It is judged that bacteria has not grown where the viable cell count is not more than 5.

\* The final concentration of an agent is half the concentration of the agent added because the agent is mixed with the bacteria in equal amounts.

Test Example 7 (Comparative test of antibacterial power of silver conjugated protein and known silver-containing antibacterial agent)

10 Comparative test of the antibacterial power of the silver  
conjugated protein ② (having a silver content of 7.0 wt%) and a  
known silver-containing antibacterial agent against  
Staphylococcus aureus was carried out under the same  
conditions as in Test Example 1.

15           The known silver-containing antibacterial agent was as follows, and silver nitrate was also tested for reference.

BIKAHM AK-LS (produced by Otsuka Chemical Co., Ltd., Japan, having a silver content of 15 wt%).

The obtained results are shown in Table 6. The numerical values in the table represent viable cell counts.

Table 6

Concen- tration of agent added (ppm)	Silver conjugated protein ②	BIKAHM AK- LS (Otsuka Chemical Co., Ltd.	Concen- tration of Ag in silver nitrate (ppm)	Silver Nitrate
Not added	$3.50 \times 10^6$	ditto	ditto	ditto
200.00	0	$\infty$	12.50	0
100.00	0	$\infty$	6.25	1
50.00	0	$\infty$	3.13	1
25.00	0	$\infty$	1.56	1
12.50	0	$\infty$	0.78	133
6.25	4	$\infty$	0.39	$\infty$
3.13	14695	$\infty$	0.20	$\infty$
1.56	$\infty$	$\infty$	0.10	$\infty$
0.78	$\infty$	$\infty$		

\* Test bacteria : Staphylococcus aureus

\* It is judged that bacteria has not grown where the viable cell count is not more than 5.

- 5 \* The final concentration of an agent is half the concentration of the agent added because the agent is mixed with the bacteria in equal amounts.

10 Test Example 8 (Comparative test of antibacterial power of silver conjugated protein and official silver protein)

Comparative test of the antibacterial power of the silver conjugated protein ② (having a silver content of 7.0 wt%) and

an official silver protein against *Staphylococcus aureus* was carried out under the same conditions as in Test Example 1.

Determination method : in accordance with the determination of the minimum bactericidal concentration (MBC) of Antibacterial Evaluation Tests For Inorganic Antibacterial Agents Such As Silver II (1995 edition, edited by the Society for the Study of Inorganic Antibacterial Agents Such As Silver)

Test bacterial : *Staphylococcus aureus* IFO 12732

Medium used : SCD medium.

The following was used as an official silver protein, and silver nitrate was also tested for reference.

Official Protein Silver (produced by MARUISHI Pharmaceutical Co., Ltd., having a silver content of 8 wt%)

The obtained results are shown in Table 7. The numerical values in the table represent viable cell counts.



[illegible]

Concen- tration of agent added (ppm)	Silver conjugated protein ②	Official Protein Silver (MARUISHI Pharmaceu- tical Co. Ltd.)	Concen- tration of Ag in silver nitrate (ppm)	Silver Nitrate
Not added	$3.50 \times 10^6$	ditto	ditto	ditto
200.00	0	0	12.50	0
100.00	0	0	6.25	1
50.00	0	0	3.13	1
25.00	0	0	1.56	1
12.50	0	128	0.78	133
6.25	4	170	0.39	$\infty$
3.13	14695	$\infty$	0.20	$\infty$
1.56	$\infty$	$\infty$	0.10	$\infty$
0.78	$\infty$	$\infty$		

\* Test bacteria : Staphylococcus aureus

\* It is judged that bacteria has not grown where the viable cell count is not more than 5.

5    \* The final concentration of an agent is half the concentration  
of the agent added because the agent is mixed with the bacteria  
in equal amounts.

It is understood from the results shown in Tables 1 to 6  
10 that the silver conjugated proteins of the present invention have  
more excellent antibacterial/antifungal activity than the known  
inorganic antibacterial agents containing silver. BIKAHM AK-  
LS, a known silver-containing antibacterial agent, used in Test

Example 7 whose results are shown in Table 6 is particularly inferior in the antibacterial/antifungal activity although its silver content is as high as 15 wt%. That is considered because silver ions are not released in a sustained manner in an appropriate amount when the antibacterial activity should be exhibited. As clearly understood from the results in Table 7, the antibacterial/antifungal activity of the silver conjugated proteins of the present invention is more excellent than that of the official protein.

10

Test Example 9 (Comparative test of fungal resistance of silver conjugated proteins and known silver-containing antibacterial agent)

Comparative test of the fungal resistance of the silver conjugated protein ①, the silver conjugated protein ②, the silver conjugated protein ③ and the known silver-containing antibacterial agent [Zeomic (tradename, produced by SHINANEN ZEOMIC CO., LTD.)] was carried out under the following conditions.

20 Determination method : in accordance with JIS K2911-1981 fungal resistance test method, particularly the following items:

- 3. Preparation for test 3.5 Spore suspension
- 4. General rule of test 4.3.2. Expression of test results

Table 1 "How to express test results"

25 6. Test of fiber products 6.2.2. Wet method

Fungi : First group : *Aspergillus niger* (FERM S-1)

Second group : *Penicillium citrinum* (FERM S-5)

Third group : *Rhizopus stolonifer* (FERM S-7)

Fourth group : *Cladosporium cladosporioides*

5 (FERM S-8)

Fifth group : *Chaetomium globosum* (FERM S-11)

Commercially available copy paper was used as paper to be coated. Coating was performed by a Baker-type applicator (produced by SASUDA SEIKI) under the condition of a coating  
10 thickness of 1 to 10  $\mu$  m. Each agent was prepared in the following concentrations. Both faces of the paper were coated, dried by airflow at 60 °C for an hour and made into a test piece (5 cm  $\times$  5 cm) having an intended coating weight.

The agent of the silver conjugated protein ① was  
15 prepared by dispersing 256 mg of the silver conjugated protein ① in 20 mL of deionized water. This agent was used for producing coated test pieces with coating weights of 1g/m<sup>2</sup>, 2g/m<sup>2</sup> and 4g/m<sup>2</sup>.

The agent of the silver conjugated protein ② was  
20 prepared by dispersing 256 mg and 1280 mg of the silver conjugated protein ② each in 20 mL of deionized water. This agent was used for producing coated test pieces with coating weights of 1g/m<sup>2</sup>, 2g/m<sup>2</sup> and 4g/m<sup>2</sup>.

The agent of the silver conjugated protein ③ was  
25 prepared by dispersing 256 mg of the silver conjugated protein

③ in 20 mL of deionized water. This agent was used for producing coated test pieces with coating weights of  $1\text{g/m}^2$ ,  $2\text{g/m}^2$  and  $4\text{g/m}^2$ .

Zeomic was dispersed in deionized water to prepare a  
 5 1% agent and a 10% agent. These agents were used for producing coated test pieces with coating weights of  $1\text{g/m}^2$ ,  $2\text{g/m}^2$  and  $4\text{g/m}^2$ .

The growth of a fungus was observed on test pieces coated with the antibacterial/antifungal agents in varied  
 10 coating weights (on both faces). Uncoated paper was tested under the same conditions as control for every test.

Figs. 3(a), 3(b) and 3(c) show the growth of a fungus with varying the coating weight of the silver conjugated protein  
 ② to  $1\text{g/m}^2$ ,  $2\text{g/m}^2$  and  $4\text{g/m}^2$ .

15 Figs. 4(a), 4(b) and 4(c) show the growth of a fungus with varying the coating weight of the silver conjugated protein  
 ③ to  $1\text{g/m}^2$ ,  $2\text{g/m}^2$  and  $4\text{g/m}^2$ .

Figs. 5(a), 5(b) and 5(c) show the growth of a fungus with varying the coating weight of Zeomic to  $1\text{g/m}^2$ ,  $2\text{g/m}^2$  and  
 20  $4\text{g/m}^2$ .

The results of the fungal resistance test are also shown in Table 8.



It is understood from the results shown in Table 8 that the silver conjugated proteins ② and ③ exhibit much more excellent fungal resistance than the known silver-containing antibacterial agent.

The silver conjugated proteins ② and ③, if used at an increased coating weight, are observed to change color due to silver. However, from the viewpoint of the fungal resistance, sufficient is a small coating weight such that a change of color does not take place. Therefore, there are no problems with the change of color.

#### Test Example 10 (Fungal resistance test of silver conjugated protein)

Pieces of paper coated with the silver conjugated protein ③ were tested on their fungal resistance in accordance with JIS K2911-1981 fungal resistance test method.

Fungi used : *Neosartorya fischeri*

Liner paper was used as paper to be coated. Coating was performed by a Baker-type applicator (produced by YASUDA SEIKI, Japan) under the condition of a coating thickness of 1  $\mu$  m or 3  $\mu$  m. Both faces of the paper were coated, dried by airflow at 60 °C for an hour and made into a test piece (5 cm  $\times$  5 cm) having a coating weight of 0.14 g/m<sup>2</sup> and 0.42 g/m<sup>2</sup>.

The obtained results are shown in Fig. 6 and Fig. 7.

Fig. 6 shows the growth of the fungus where the silver

conjugated protein ③ was applied on the liner paper at a coating weight of 0.14g/m<sup>2</sup>.

Fig. 7 shows the growth of the fungus where the silver conjugated protein ③ was applied on the liner paper at a coating weight of 0.42 g/m<sup>2</sup>.

The results of the fungal resistance test are also shown in Table 9.

Table 9

	Silver conjugated protein ③			Silver conjugated protein ③			Control
Coating weight	0.14 g/m <sup>2</sup>			0.42 g/m <sup>2</sup>			Not coated
Test piece	No.1	No.2	No.3	No.1	No.2	No.3	No.1
Judgement	3	3	3	3	3	3	1

1 : The area where hyphae grew exceeded one-third of the total area.

2 : The area where hyphae grew did not exceed one-third of the total area.

3 : The growth of hyphae was not recognized.



It is understood from Fig. 6, Fig. 7 and the results shown in Table 9, the silver conjugated protein ③ also provides good fungal resistance to liner paper whose both faces are coated at coating weights of 0.14 g/m<sup>2</sup> and 42 g/m<sup>2</sup>.

Test Example 11 (Fungal resistance test of silver conjugated proteins)

Pieces of paper coated with the silver conjugated proteins ⑤ to ⑦ were tested on their fungal resistance in accordance with JIS K2911-1981 fungal resistance test method.

Liner paper was used as paper to be coated. Coating was performed by a Baker-type applicator (produced by YASUDA SEIKI) under the condition of a coating thickness of 1 μm or 3 μm. Both faces of the paper were coated, dried by airflow at 60 °C for an hour and made into a test piece (5 cm × 5 cm) having an intended coating weight.

Fungi used : Spore suspension of *Neosartorya fischeri*

The growth of the fungi was observed with varying the coating weight of the antibacterial/antifungal agents (applied on both faces) on the test pieces. Uncoated paper was tested under the same conditions as control for every test.

The obtained results are shown in Table 10.

Table 10

	Silver conjugated proteins ⑤			Silver conjugated proteins ⑥			Silver conjugated proteins ⑦		
Coating weight	0.15 g/m <sup>2</sup>	0.45 g/m <sup>2</sup>	0.90 g/m <sup>2</sup>	0.15 g/m <sup>2</sup>	0.45 g/m <sup>2</sup>	0.90 g/m <sup>2</sup>	0.15 g/m <sup>2</sup>	0.45 g/m <sup>2</sup>	0.90 g/m <sup>2</sup>
1st judgement	2	2	3	2	3	3	1	2	2
2nd judgement	2	3	3	3	3	3	2	2	3
3rd judgement	2	3	3	3	3	3	2	2	3
									Not Coated
									1
									1
									1

- 1 : The area where hyphae grew exceeded one-third of the total area.  
 2 : The area where hyphae grew did not exceed one-third of the total area.  
 3 : The growth of hyphae was not recognized.

It is understood from Table 10 that the test pieces coated with the silver conjugated proteins ⑤ to ⑦ have a judgement of 2 or more and have good fungal resistance while the uncoated test pieces have a judgement of 1. The silver conjugated proteins exhibit a remarkable effect when used particularly at a coating weight of 0.45 g/m<sup>2</sup> or more.

Test Example 12 (Fungal resistance test of silver conjugated protein)

Pieces of paper coated with the silver conjugated protein ③ were tested on their fungal resistance in accordance with JIS K2911-1981 fungal resistance test method.

Liner paper was used as paper to be coated. Coating was performed by a Baker-type applicator (produced by YASUDA SEIKI) under the condition of a coating thickness of 1 μm or 3 μm. Both faces of the paper were coated, dried by airflow at 60 °C for an hour and made into a test piece (5 cm × 5 cm) having an intended coating weight.

Fungi used : the same spore suspension as used in Test Example 9

The obtained results are shown in Fig. 8 and Fig. 9.

Fig. 8 shows the growth of the fungus where the silver conjugated protein ③ was applied on the liner paper at a coating weight of 0.14g/m<sup>2</sup>.

Fig. 9 shows the growth of the fungus where the silver



Table 11

	Silver conjugated protein ③			Silver conjugated protein ③			Control
Coating weight	0.14 g/m <sup>2</sup>			0.42 g/m <sup>2</sup>			Not coated
Test piece	No.1	No.2	No.3	No.1	No.2	No.3	No.1
Judgement	2	3	3	3	3	3	1

- 1 : The area where hyphae grew exceeded one-third of the total area.
- 2 : The area where hyphae grew did not exceed one-third of the total area.
- 3 : The growth of hyphae was not recognized.

It is understood from Fig. 8, Fig. 9 and the results shown in Table 11 that the silver conjugated protein ③ also provides good fungal resistance to liner paper whose both faces are coated at coating weights of 0.14 g/m<sup>2</sup> and 42 g/m<sup>2</sup>.

Test Example 13 (Observation of paper coated with silver conjugated proteins by scanning electronic microscope)

The surface of the copy paper coated with the silver conjugated protein ①, the silver conjugated protein ③ and Zeomic, a known silver-containing antibacterial agent, in Test Example 9 was observed by a scanning electronic microscope.

The obtained results are shown in Fig. 10 to Fig. 12.

Fig. 10 shows the state of the surface of the copy paper coated with the silver conjugated protein ①.

Fig. 11 shows the state of the surface of the copy paper coated with the silver conjugated protein ③.

Fig. 12 shows the state of the surface of the copy paper coated with Zeomic.

It is observed in Fig. 10 and Fig. 11 that the silver conjugated proteins ① and ③ adhere to the surface of the paper like a film filling spaces between pulp fibers. On the other hand, it is observed in Fig. 12 that the known silver-containing antibacterial agent Zeomic adheres to the surface of paper as fine particles. However, these particles easily fall away by being touched with fingers.

For this reason, it is understood that inorganic silver antibacterial agent, when used by being applied onto the surface of paper, needs a resin binder (an adhesive or a binder). In contrast, the silver conjugated proteins of the present invention have properties such as a film-forming property, a sticking property and the like which come from the raw material proteins. Therefore, the silver conjugated proteins can be applied to paper without using adhesives or binders.

Test Example 14 (KBB size degree test of paper coated with silver conjugated proteins)

The KBB size degree test was carried out on the copy paper coated with the silver conjugated protein ①, the silver conjugated protein ③ and the known silver-containing antibacterial agent Zeomic in Test Example 9, in accordance with JIS P8122. The test was performed by use of an automatic KBB size degree measuring apparatus produced by YASUDA SEIKI.

The obtained results are shown in Table 12.

Table 12

(uncoated paper) commercially available copy paper : KBB size degree 86.9 seconds in average

Coating weight of silver conjugated protein ① (g/m <sup>2</sup> )	KBB size degree (sec.)	Coating weight of silver conjugated protein ③ (g/m <sup>2</sup> )	KBB size degree (sec.)	Coating weight of Zeomic (g/m <sup>2</sup> )	KBB size degree (sec.)
1	56.7	1	102	1	115.8
2	1600.0	2	125	2	116.4
5	3800.0	4	162	10	122.0

Note : the coating weight is the sum of the coating weights on both faces



It is understood from the results shown in Table 12 that the copy paper coated with the silver conjugated protein ① and the silver conjugated protein ③ has a larger size degree as the coating weight increases. The silver conjugated protein ①, especially, provides a remarkable increase in the size degree. On the other hand, the copy paper coated with Zeomic has an increase in the size degree, though the increase is small. That is because a lot of Zeomic particles adhere to the surface of the paper and, as the thickness of the particles increases, more time is required for penetration of an electrolytic liquid, which is apparently observed as an increase in the size degree.

Test Example 15 (Test of antibacterial activity of liner paper coated with silver conjugated proteins)

Pieces of paper coated with the silver conjugated proteins ⑤ to ⑦ were tested on their antibacterial activity in accordance with JIS L1902.

For the test, the coated test pieces in Test Example 11 were cut into 18 mm × 18 mm beforehand and used as test pieces. *Staphylococcus aureus* IFO 12732, a bacterium used, was inoculated into a SCD agar medium and cultured at 37 °C for 24 to 48 hours. After culture, a loopful of the resulting culture was collected, inoculated into an L-shaped tube fed with 5 mL of the SCD medium and cultured at 37 °C for 18 hours. The resulting culture was diluted 10 fold with a sterilized

physiological saline and diluted with the SCD medium so that the absorbance at 660 nm was 0.1. Thereby the viable cell count became  $1 \text{ to } 2 \times 10^8 / \text{mL}$ . The resulting liquid was diluted with a 20-fold dilution liquid of the SCD medium ice-cooled so that the viable cell count became  $1 \pm 0.3 \times 10^5 / \text{mL}$ , thereby obtaining test bacteria.

Six pieces of uncoated paper and three pieces of coated paper were prepared. The test pieces were put at the bottom of vials autoclaved at  $121^\circ \text{C}$  for 15 minutes. Thereafter, 0.2 mL of the prepared test bacteria were inoculated uniformly on several sites of each of the test pieces and cultured at  $37^\circ \text{C}$  for 18 hours.

After culture, 20 ml of physiological saline were fed into each vial and stirred (for 5 seconds, 5 times) by a test tube oscillator to wash out remaining bacteria.

One mL of each washing was taken and added to 9 mL of sterilized physiological saline. The resulting dilution liquid, 1 mL, was taken and added to 9 mL of sterilized physiological saline to prepare a dilution liquid. The prepared dilution liquid was fed in two laboratory dishes, 1 mL each, to which the SCD agar medium was introduced. Culture was carried out at  $37^\circ \text{C}$  for 48 hours. The number of colonies was counted after culture to judge the antibacterial effect of the coated test pieces.

The obtained results are shown in Tables 13 to 16. In the tables, "aE + b" represents " $a \times 10^b$ ."

Table 13

(Vial cell counts in uncoated test pieces immediately after inoculation)

Sample name	No. of test piece	Lab. dish for cell count	Cell count in lab. dish (number/mL)	Vial cell count in each test piece (number/mL)	Average cell count in uncoated test pieces (number/mL)
Uncoated sample	1	1-1	401	8.5E + 4	8.0E + 4
		1-2	452		
Uncoated sample	2	2-1	417	8.3E + 4	
		2-2	414		
Uncoated sample	3	3-1	355	7.2E + 4	
		3-2	360		

Dilution R was 10-fold when the cell count was measured.

Table 14

(Vial cell counts in uncoated test pieces after 18 hours' culture)

Sample name	No. of test piece	Lab. dish for cell count	Cell count in lab. dish (number/mL)	Vial cell count in each test piece (number/mL)	Average cell count in uncoated test pieces (number/mL)
Uncoated sample	1	1-1	261	5.8E + 4	5.9E + 4
		1-2	316		
Uncoated sample	2	2-1	265	6.0E + 4	
		2-2	337		

Dilution R was 100-fold when the cell count was measured.

Table 15  
(Vial cell counts in coated test pieces after 18 hours' culture)

Sample name	No. of test piece	Lab. dish for cell count	Cell count in lab. dish (number/mL)	Cell count in test piece (number/mL)	Bacterio-static activity value	Bacterio-cidal activity value	Antivirability
Silver conjugated protein ⑤ Coating weight of 0.15 g/m <sup>2</sup>	1	1-1	0	2E + 3	2.5	1.6	2.5
		1-2	1				
	2	2-1	0	< 1E + 3	> 2.8	> 1.9	2.8
		2-2	0				
	3	3-1	16	5.5E + 4	1.0	0.16	1.0
		3-2	39				
Silver conjugated protein ⑤ Coating weight of 0.45 g/m <sup>2</sup>	1	1-1	0	2E + 3	2.5	1.6	2.5
		1-2	2				
	2	2-1	4	4E + 3	2.2	1.3	2.2
		2-2	4				
	3	3-1	0	< 1E + 3	> 2.8	> 1.9	> 2.8
		3-2	0				

(continued Table 15)

Silver conjugated protein ⑤	1	1-1	4	3E + 3	2.3	1.4	2.3
		1-2	2				
Coating	2	2-1	0	< 1E + 3	> 2.8	> 1.9	> 2.8
		2-2	0				
weight of 0.90 g/m <sup>2</sup>	3	3-1	23	3.6E + 4	1.2	0.35	1.2
		3-2	13				
Silver conjugated protein ⑥	1	1-1	1	3E + 3	2.3	1.4	2.3
		1-2	2				
0.15 g/m <sup>2</sup>	2	2-1	0	< 1E + 3	> 2.8	> 1.9	> 2.8
		2-2	0				
	3	3-1	1	6E + 3	2.0	1.1	2.0
		3-2	5				

(continued Table 15)

Silver conjugated protein ⑥ Coating weight of 0.45 g/m <sup>2</sup>	1	1-1	0	1E + 3	2.8	1.9	2.8
		1-2	1				
	2	2-1	0	1E + 3	2.8	1.9	2.8
		2-2	1				
	3	3-1	17	2.8E + 4	1.3	0.46	1.3
		3-2	11				
Silver conjugated protein ⑥ Coating weight of 0.90 g/m <sup>2</sup>	1	1-1	0	< 1E + 3	> 2.8	> 1.9	> 2.8
		1-2	0				
	2	2-1	0	< 1E + 3	> 2.8	> 1.9	> 2.8
		2-2	0				
	3	3-1	6	8E + 3	1.9	1.0	1.9
		3-2	2				

Dilution R was 100-fold when the cell count was measured.

Table 16

(continued Table 15)

Sample name	No. of test piece	Lab. dish for cell count	Cell count in lab. dish (number/mL)	Cell count in test piece (number/mL)	Bacterio-static activity value	Bacterio-cidal activity value	Antivirability
Silver conjugated protein ⑦ Coating weight of 0.15 g/m <sup>2</sup>	1	1-1	3	6E + 3	2.0	1.1	2.0
		1-2	3				
	2	2-1	3	7E + 3	1.9	1.1	1.9
		2-2	4				
	3	3-1	28	4.5E + 4	1.1	0.25	1.1
		3-2	17				
Silver conjugated protein ⑦ Coating weight of 0.45 g/m <sup>2</sup>	1	1-1	52	1.5E + 5	0.59	0.27	0.59
		1-2	99				
	2	2-1	3	3E + 3	2.3	1.4	2.3
		2-2	0				
	3	3-1	2	5E + 4	1.1	0.20	1.1
		3-2	3				



(continued Table 16)

Silver conjugated protein ⑦	1	1-1	12	2.1E + 4	1.4	0.58	1.4
		1-2	9				
Coating	2	2-1	12	1.2E + 4	1.7	0.82	1.7
		2-2	0				
weight of 0.90 g/m <sup>2</sup>	3	3-1	9	2.3E + 4	1.4	0.54	1.4
		3-2	14				
Not coated	1	1-1	261	5.8E + 5	0	-	0
		1-2	316				
	2	2-1	265	6.0E + 5	0	-	0
		2-2	337				

Dilution R was 100-fold when the cell count was measured.

The antibacterial effect was judged by the bacteriostatic activity value, the bacteriocidal activity value and logarithmic reduction rate obtained by the following formulae.

$$\text{Bacteriocidal activity value } L = Ma - Mc$$

5       $\text{Bacteriostatic activity value } S = Mb - Mc$

Ma : Common logarithmic value of the vial cell count (average of three test pieces) on uncoated test pieces immediately after the inoculation

Mb : Common logarithmic value of the vial cell count  
10 (average of two test pieces) on uncoated test pieces after 18 hours' culture

Mc : Common logarithmic value of the vial cell count on coated test pieces after 18 hours' culture

$$\text{Logarithmic reduction rate} = \log (B/A) - \log (C/A)$$

15      A : Vial cell count (average of three test pieces) on uncoated test pieces immediately after the inoculation

B : Vial cell count (average of two test pieces) on uncoated test pieces after 18 hours' culture

C : Vial cell count on coated test pieces after 18  
20 hours' culture

In most of the test pieces coated with the silver conjugated proteins ⑤ to ⑦, the cell count in the laboratory dishes was suppressed to zero. The bacteriostatic activity value, the bacteriocidal activity value and logarithmic reduction  
25 rate were, about two or more, one or more and two or more,

respectively.

From the above, the antibacterial activity was observed in the coated test pieces, as contrasted with the uncoated test pieces.

5

The water-insoluble silver conjugated protein of the present invention is formed of a silver salt and a water-soluble protein having an active thiol group content of 0.1 to 200  $\mu$  mol/g.

10

The water-insoluble silver conjugated protein of the present invention is extremely safe because it is made from a protein used in the fields of food and cosmetics. Silver, having the antibacterial/antifungal activity, does not separate from the protein easily, and the silver content is maintained at a high level.

15

Therefore, the water-insoluble silver conjugated protein of the present invention is useful as an antibacterial/antifungal agent in various fields, particularly useful for antibacterial/antifungal paper.